TOXICOLOGICAL SCIENCES **106(1)**, 93–102 (2008)
doi:10.1093/toxsci/kfn150
Advance Access publication July 27, 2008

Environmentally Relevant Concentrations of 17α-Ethinylestradiol (EE2) Interfere With the Growth Hormone (GH)/Insulin-Like Growth Factor (IGF)-I System in Developing Bony Fish

Natallia Shved,* Giorgi Berishvili,* Jean-François Baroiller,† Helmut Segner,‡ and Manfred Reinecke*,[1]

*Division of Neuroendocrinology, Institute of Anatomy, University of Zürich, Winterthurerstr 190, CH-8057 Zürich, Switzerland; †Centre de Coopération Internationale en Recherche Agronomique pour le Développement-Elevage et Médecine Vétérinaire Tropicale, Unité Propre de Recherché 20, 34398 Montpellier, France; and ‡Centre for Fish and Wildlife Health, University of Bern, Länggass-Str 122, CH-3001 Bern, Switzerland

Received March 27, 2008; accepted July 19, 2008

The aim of this study was to evaluate whether effects of environmental estrogens on fish growth and reproduction may be mediated via modulating the growth hormone (GH)/insulin-like growth factor I (IGF-I) system. To this end, developing male and female monosex populations of tilapia were exposed to 17α-ethinylestradiol (EE2) at 5 and 25 ng EE2/l water from 10-day postfertilization (DPF) until 100 DPF. Under exposure to both EE2 concentrations, sex ratio shifted toward more females and body length, and weight were significantly reduced in males. The growth-reducing effect was associated with significant changes in hepatic IGF-I expression, both in males and females and with significant alterations of IGF-I mRNA and GH mRNA in the brain. The changes in IGF-I and GH mRNA were accompanied by altered estrogen receptor α (ERα) expression in brain and liver. These findings point to an influence of estrogenic exposure on the endocrine GH/IGF-I axis. In addition, the EE2 treatment resulted in significant changes of ERα and IGF-I expression in ovaries and testis, suggesting that the estrogens interact not only with the endocrine but also with the autocrine/paracrine part of the IGF-I system. Overall, our results provide evidence that EE2 at environmentally relevant concentrations is able to interfere with the GH/IGF-I system in bony fish and that the impairing effects of estrogens reported on fish growth and reproductive functions may rather result from a cross talk between the sex steroid and the IGF-I system than be toxicological.

Key Words: **IGF-I; growth hormone; development; liver; brain; ovary; testis; teleost.**

Endocrine disrupting compounds (EDCs) have the potential to disrupt internal homeostasis and hormone-controlled physiological processes such as development, growth, stress responses, sexual differentiation, or reproduction. Many EDCs end up in the aquatic environment and therefore primarily affect aquatic organisms including fish. Numerous effects of EDCs on fish from all over the world have been described, such as deformities and defects in fish larvae from the North Sea (Dethlefsen *et al.*, 1996), feminization of male fish near sewage treatment outlets in the United Kingdom (Gross-Sorokin *et al.*, 2006; Sumpter and Jobling, 1995), significant alterations of the steroid system of carps from the Ebro River (Lavado *et al.*, 2004), and impaired growth in male mosquito fish in Western Australia (Game *et al.*, 2006). Furthermore, exposure to estrogenic compounds at low concentrations can lead to growth impairment, as it has been shown for rainbow trout (Ashfield *et al.*, 1998) and zebra fish (Schäfers *et al.*, 2007). However, it is unknown whether the mechanisms underlying the growth impairment represents a general toxicological stress rather than an endocrine disrupting effect.

Insulin-like growth factor I (IGF-I) is crucially involved in the regulation of growth, differentiation, and reproduction by selectively promoting mitogenesis and differentiation (Jones and Clemmons, 1995; Reinecke and Collet, 1998). Among the nonmammalian classes of vertebrates, bony fish are the mostly studied with respect to IGF-I (Reinecke *et al.*, 2005; Wood *et al.*, 2005) mainly due to their unique development from the larval to the adult life, their high growth potential, and to their importance in aquaculture. As in mammals, IGF-I is mainly produced in fish liver—the principal source of the circulating (endocrine) IGF-I—under the influence of growth hormone (GH). In addition, IGF-I is expressed in parenchymal cells of numerous extrahepatic sites of adult (Reinecke *et al.*, 1997) and developing (Berishvili *et al.*, 2006a,b; Perrot *et al.*, 1999; Radaelli *et al.*, 2003) fish and most likely stimulates organ-specific functions by paracrine/autocrine mechanisms. There is increasing evidence that GH stimulates the expression of IGF-I not only in fish liver but also in extrahepatic sites (Biga *et al.*, 2004; Eppler *et al.*, 2007; Vong *et al.*, 2003).

From *in vivo* and *in vitro* studies with adults of different fish species, initial evidence has been provided on an interaction between the estrogen and IGF-I system. In particular, 17β-estradiol (E2) appears to be able to downregulate hepatic IGF-I expression (Carnevali *et al.*, 2005; Davis *et al.*, 2007; Filby *et al.*, 2006; Lerner *et al.*, 2007; Riley *et al.*, 2004) or serum IGF-I (Arsenault *et al.*, 2004; Davis *et al.*, 2007;

[1] To whom correspondence should be addressed. Fax: +41 44 635-5702. E-mail: reinecke@anatom.uzh.ch.

McCormick *et al*., 2005). In a recent study on tilapia, *Oreochromis niloticus*, we could show that early life exposure to elevated concentrations of 17α-ethinylestradiol (EE2), a major constituent of contraceptive pills, has long-lasting consequences on growth, IGF-I serum levels, and IGF-I expression in liver as well as in gonads (Shved *et al*., 2007). These data suggest that estrogen effects on the GH/IGF-I system may be more pronounced during fish development, that is, in the phase of rapid growth as well as of sexual differentiation and gonad development.

In the previous study, we used rather high EE2 concentrations (Shved *et al*., 2007) in order to establish principally whether the estrogen and GH/IGF-I system do interact. The effects observed with this approach, however, may reflect a general toxic stress situation rather than a specific endocrine disrupting mode of action. The aim of the present study was to investigate whether low estrogen exposure at concentrations at environmentally observed levels are able to disrupt the GH/IGF-I system and growth in developing tilapia. Thus, our hypothesis was that the EE2 impact on the GH/IGF-I system as observed at high estrogen doses is still valid at low, environmentally relevant concentrations.

It is known that EDC exposure of fish during critical stages of gonad development can induce permanent, organizational changes in sexual differentiation; however, currently very few studies have addressed whether early life exposure to EDCs, particularly estrogens, can permanently alter functioning of other physiological systems in fish (Lerner *et al*., 2007; Milston *et al*., 2003).

Concentrations of EE2 in the aquatic environment in the low nanogram per liter range have been observed in numerous countries. In Germany, concentrations of EE2 in rivers were up to 5 ng/l and in waste water treatment plants effluents ranged from 9 (Kuch and Ballschmiter, 2001) to 15 ng/l (Ternes *et al*., 1999). Similarly, in France and the Netherlands, 3–4 ng EE2/l were measured in coastal water and rivers and 4–8 ng EE2/l in effluents (Belfroid *et al*., 1999; Cargouët *et al*., 2004). EE2 concentrations in effluents in the United Kingdom were around 7 ng/l (Desbrow *et al*., 1998) and in a river in Taipei around 15 ng EE2/l (Chen *et al*., 2007). United States Stream Data 1999–2000 give the EE2 content in 139 streams across 30 states (Kolpin *et al*., 2002) between 5 and 273 ng EE2/l, and in Canada, up to 42 ng EE2/l were reported in effluents (Ternes *et al*., 1999).

In the present study, female and male "monosex" populations of tilapia (*O. niloticus*) were experimentally exposed to 5 and 25 ng EE2/l—concentrations well within the range found in the environment—from 10-day postfertilization (DPF) until 100 DPF, a period covering the sensitive period of gonadal development and ranging until the end of puberty. Male and female fish were investigated at 30, 50, 75, and 100 DPF. The question was assessed whether EE2 exposure during development exerts acute and lasting effects on the expression of GH mRNA in pituitary and of IGF-I mRNA in liver and extrahepatic sites, that is, gonad and brain. Finally, the expression of estrogen receptor α (ERα) that is considered to be the ER subtype most responsive to estrogen (e.g., Filby *et al*., 2007; Marlatt *et al*., 2008; Menuet *et al*., 2004) was also measured in the organs studied.

MATERIALS AND METHODS

Generation and maintenance of fish. To allow sex-differentiated analysis of very early stages, monosex breedings of *O. niloticus* generated as described before (Baroiller *et al*., 1995, 1999) were investigated. In brief, suitable sires from sex-reversed fish were selected by progeny testing and then used to produce monosex populations that were used for our experiments. Fish were kept at the Aquaculture Experimental Facilities of CIRAD (Montpellier, France) where also the exposure experiments were performed.

Experimental conditions. Two monosex populations XX and XY ($n = 400$ each) of tilapia were incubated in 40-l aquaria at 27°C ± 1°C during a period of 10–100 DPF with EE2 (Sigma, Deisenhofen, Germany) at the nominal concentrations of 5 or 25 ng/l and with 0.4% ETOH in water (solvent control). Each group was run in duplicates. Prior to treatment, aquaria were saturated with EE2 at a concentration of 1000 ng/l during 1 week. During the exposure, water in all aquaria (controls, 5 ng EE2/l, 25 ng EE2/l) was renewed every 48 h. The EE2 concentrations in all aquaria were repetitively determined after 36 h.

Fish sampling and tissue preparation. At the age of 30, 50, 75, and 100 DPF fish were anesthetized with 2-phenoxy-ethanol (Sigma) added to water, and weight and length were measured. With the older stages (75 and 100 DPF), sex was additionally individually assured at sampling by external inspection of the papilla and internal inspection of the gonads. Tissue specimens (liver, brain, gonads) were excised and transferred into 1.5 ml of RNAlater (Ambion, Austin, TX), kept at 4°C and stored at − 20°C until use. Total RNA was extracted using TRIzol reagent (Invitrogen, Merelbeke, Belgium), resuspended in diethylpyrocarbonate-treated water, and photospectrometrically quantified. The following sample numbers were used for real-time PCR: control (male: 30 DPF $n = 8$, 50 DPF $n = 12$, 75 DPF $n = 11$, 100 DPF $n = 12$; female: 30 DPF $n = 10$, 50 DPF $n = 10$, 75 DPF $n = 11$, 100 DPF $n = 12$) and EE2 exposed (5 ngEE2/l, male: 30 DPF $n = 8$, 50 DPF $n = 8$, 75 DPF $n = 7$, 100 DPF $n = 8$; female: 30 DPF $n = 9$, 50 DPF $n = 8$, 75 DPF $n = 10$, 100 DPF $n = 8$; 25 ng EE2/l, male: 30 DPF $n = 7$, 50 DPF $n = 7$, 75 DPF $n = 8$, 100 DPF $n = 8$; female: 30 DPF $n = 7$, 50 DPF $n = 8$, 75 DPF $n = 9$, 100 DPF $n = 8$).

Solid-phase extraction/enzyme-linked immunosorbent assay. To assess the actual EE2 concentration during the experiment, water samples were collected from the control and exposure aquaria. The EE2 ELISA kit (Ecologiene, Japan, purchased from Biosense Laboratories, Bergen, Norway) was used to detect EE2 in water samples. In order to concentrate the EE2 content to amounts detectable by the assay, water samples were subjected to solid-phase extraction, according to Ternes *et al*. (1999). In all, 100 mg LiChrolut EN and 200 mg RP-18 cartridges (Merck, Zug, Switzerland) were first conditioned with the series of passing—hexane, acetone, methanol, and water (pH 3.0). Prefiltered water samples (200 ml) were loaded under gentle vacuum pressure (1l/1h). Loaded cartridges were dried under nitrogen flow for 60 min. Elution was performed with 4 ml acetone in silanized amber vials (Supelco, Gland, Switzerland). Acetone was evaporated to dryness under nitrogen stream, and EE2 was reconstituted in 0.5 ml ethanol. Resulting samples were analyzed by enzyme-linked immunosorbent assay.

Design of primers and probes for real-time PCR. Based on the mRNA sequences of *O. niloticus* β-actin as housekeeping gene (Hwang *et al*., 2003), *O. mossambicus* IGF-I (Reinecke *et al*., 1997), *O. niloticus* GH (Ber and Daniel, 1992), and *O. niloticus* ERα (Chang *et al*., 1999), primers (β-actin: sense GCCCCACCTGAGCGTAAATA, antisense AAAGGTGGACAGGAGGCCA, probe TCCGTCTGGATCGGAGGCTTCATC; IGF-I: sense

TCTGTGGAGAGCGAGGCTTT, antisense CACGTGACCGCCTTGCA, probe ATTTCAATAAACCAACAGGCTATGGCCCCA; GH: sense TCGACAAACACGAGACGCA, antisense CCCAGGACTCAACCAGTCCA, probe CGCAGCTCGGTCCTGAAGCTG, and ERα: sense CAAGTGGTGGAGGAGGAAGATC, antisense CTCAGCACCCTGGAGCAG, probe CTGATCAGGTGCTCCTC) were created as described previously (Caelers *et al*., 2004; Shved *et al*., 2007). Oligonucleotides were designed with Primer Express software version 1.5 (PE Biosystems, Foster City, CA).

Quantitation of IGF-I and ERα expression by two-step real-time reverse transcriptase-PCR TaqMan system. Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Catalys AG, Wallisellen, Switzerland). Single-stranded cDNA was synthesized from 800 ng total RNA using 1× TaqMan reverse transcription buffer, $MgCl_2$ (5.5mM), 1.25 U/μl of moloney murine leukemia virus reverse transcriptase, 2.5μM of random hexamers primers, 0.4 U/μl ribonuclease inhibitor, 500μM each dNTP (Applied Biosystems, Rotkreuz, Switzerland) for 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. In all, 2 μl cDNA obtained from 10 ng/μl total RNA were subjected, in duplicates, to real-time PCR using an Absolute QPCR low ROX Mix (ABgene, Epsom, UK) including Thermo-Start DNA polymerase, 300nM of each primer, and 150nM of the fluorogenic probe. Amplification was performed with a total reaction volume of 10 μl in a MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems). Reactions were run on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with the following conditions: 95°C for 15 min of the enzyme activation followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Relative quantification of treatment effects using the ΔΔCT method. The relative gene expression ratios between EE2-exposed and control fish were calculated using the comparative C_T method ($\Delta\Delta C_T$ method). For validation of quantitative PCR assays, the C_T values were plotted against the logarithms of the dilution factors and the slope was determined. Relative changes induced by EE2 exposure were calculated using the formula $2^{-\Delta\Delta C_T}$ with $\Delta C_T = C_T$ (target gene) − C_T (internal control) and $\Delta\Delta C_T = \Delta C_T$ (treated group) − ΔC_T (untreated control). Thus, all experimental data are expressed as an *n*-fold difference relative to the calibrator (untreated control). Statistical significance was calculated using Mann-Whitney rank sum test with an exact *p* value. Statistical analyses were performed with GraphPad Prism 4.

RESULTS

Actual EE2 Concentrations in the Experimental Tanks

Measurements of the mean actual EE2 concentrations revealed in controls 0.009 ± 0.013 ng EE2/l, in the 5-ng EE2/l aquaria 4.34 ± 0.85 ng EE2/l, and in the 25-ng EE2/l aquaria 16.23 ± 5.74 ng EE2/l.

Sex Ratio

In our study, we have used so-called monosex populations of tilapia. In untreated controls, the percentage of females in the female monosex group amounted to about 76% and that of males in the male monosex group to 80%. A population with 75% or more fish of the same sex is considered to be monosex (D'Cotta *et al*., 2001). Thus, our percentage is well within the range that can be achieved in tilapia (Baroiller *et al*., 1995) because sex chromosome–linked genetical factors determine sex largely but not exclusively, and the development of final sex phenotype is influenced by autosomal genetic factors in combination with the hormonal, behavioral, and environmental cues (Baroiller *et al*., 1999; Devlin and Nagahama 2002).

TABLE 1
Percentage of Females and Males in the Respective Monosex Fish Populations

Monosex tilapia	Female (%)	CI[a] (%)	p^b	Male (%)	CI[a] (%)	p^b
Control	76.2	61.3–86.7		80.0	70.8–87.0	
5 ng EE2/l	94.2	83.8–98.6	0.0160	75.0	63.1–83.2	0.4235
25 ng EE2/l	95.1	83.0–99.5	0.0258	70.2	60.3–80.4	0.2010

Note. CI, confidence interval.
[a]CIs are calculated using modified Wald method (Agresti and Coull 1998) using GraphPad QuickCalc.
[b]Significances of sex ratio changes are calculated using Fisher's exact test using GraphPad Prism.

Both, the female and male monosex fish populations exhibited a higher percentage of females after exposure to both 5 and 25 ng EE2/l, the changes being significant in female monosex (Table 1).

Body Length and Weight

Exposure to EE2 affected growth more pronouncedly in male than in female fish. In male fish (Fig. 1a–c), from 50 DPF on (i.e., 40 days after start of exposure), both body length and weight were significantly reduced in fish exposed either to 5 or 25 ng EE2/l. At the end of the experiment (100 DPF), body length of males (Fig. 1a) was reduced by about 9.5 (5 ng EE2/l) and 11% (25 ng EE2/l) and body weight (Fig. 1c) by about 25% at both EE2 concentrations (Fig. 1). In female fish (Figs. 1d–f), both parameters showed only a trend to decrease. Here at 100 DPF, body length (Fig. 1d) was reduced by about 3 (5 ng EE2/l) and 6.2% (25 ng EE2/l) and body weight (Fig. 1f) by about 14% at 25 ng EE2/l.

Liver IGF-I and ERα Gene Expression

In both sexes, an initial (30 DPF) significant upregulation of IGF-I mRNA levels (5 ng EE2/l, male: 2.82-fold, $p = 0.0004$; female: 2.43-fold, $p = 0.003$; 25 ng EE2/l, male: 1.74-fold, $p = 0.05$, female: 2.16-fold, $p = 0.006$) under both EE2 concentrations and in both sexes was observed (Figs. 2a and 2c). This was accompanied by elevated hepatic ERα levels (Figs. 2b and 2d) in both males and females at 5 ng EE2/l (male: 1.77-fold, $p = 0.003$; female: 2.24-fold, $p = 0.0007$) and in females (Fig. 2d) also at 25 ng EE2/l (2.66-fold, $p = 0.0007$).

During prolonged exposure (i.e., at 50, 75, and 100 DPF), the initial EE2-related upregulation of liver IGF-I mRNA changed into a downregulation. In males, IGF-I gene expression was significantly reduced under 25 ng EE2/l (− 2.38-fold, $p = 0.0003$) at 50 DPF (Fig. 2a) and with both EE2 concentrations at all subsequent time points. Changes in IGF-I gene expression were paralleled by significant downregulations of ERα gene expression levels (Fig. 2b) at 75 DPF (5 ng

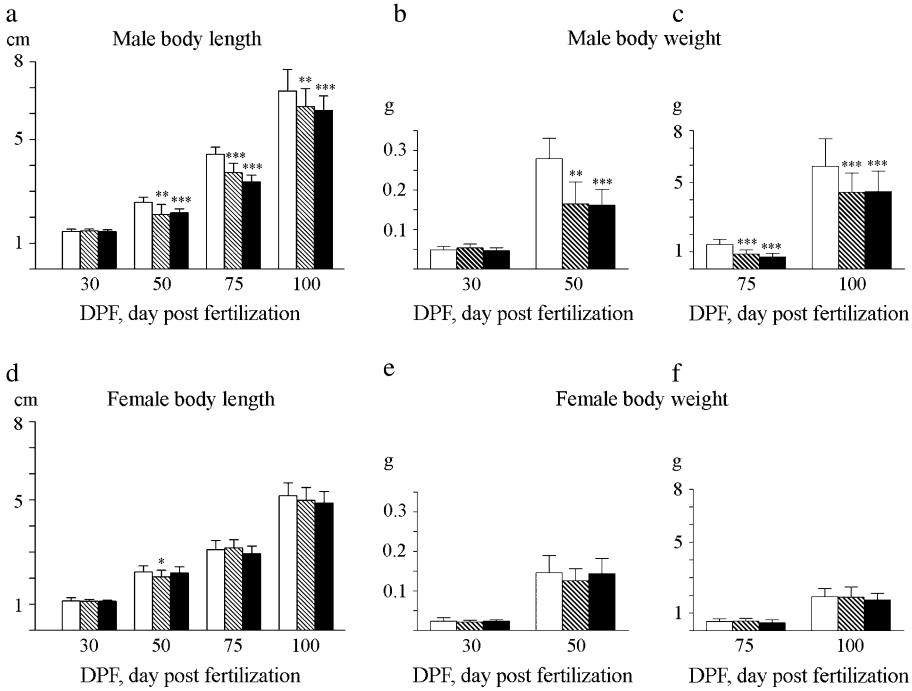

FIG. 1. Influence of EE2 exposure on somatic indices. Body length in centimeters (a, d) and weight in grams (b, c, e, f) in male (a–c) and female (d–f). White columns: control, gray columns: 5 ng EE2/l, black columns: 25 ng EE2/l. The x-axis shows stages in DPF. Bars represent mean values with SD. Significance levels: $*p < 0.05$, $**p < 0.005$, and $***p < 0.0001$.

EE2/l: − 1.64-fold, $p = 0.0047$; 25 ng EE2/l: − 2.7-fold, $p = 0.0003$) and 100 DPF (5 ng EE2/l: − 2.08-fold, $p = 0.0082$).

Similar dynamics like in males, although delayed, were observed in females: suppressed IGF-I mRNA levels (Fig. 2c) occurred in females at 75 (25 ng EE2/l: − 1.41-fold, $p = 0.0093$) and 100 DPF (5 ng EE2/l: − 1.85-fold, $p = 0.015$). The observed lowered IGF-I mRNA levels, in part, coincided with suppression (100 DPF, 25 ng EE2/l: − 1.59-fold, $p = 0.05$) of ERα mRNA (Fig. 2d).

Brain IGF-I and ERα Gene Expression

In male brains, IGF-I mRNA levels were significantly suppressed at 30 (− 1.41-fold, $p = 0.0499$) and 50 DPF (− 1.72-fold, $p = 0.0148$) at the higher EE2 concentration (25 ng EE2/l) but returned to control levels at 75 DPF (Fig. 3a). ERα mRNA was significantly elevated at 30 DPF at both concentrations (5 ng EE2/l: 1.76-fold, $p = 0.0499$; 25 ng EE2/l: 1.73-fold, $p = 0.0379$) and at 75 DPF at 5 ng EE2/l (1.65-fold, $p = 0.0205$) (Fig. 3b). At end of the experimental period (100 DPF), a significant downregulation (− 1.89-fold, $p = 0.0011$) of ERα mRNA was observed under 5 ng EE2/l (Fig. 3b).

In female brains, IGF-I gene expression levels (Fig. 3c) revealed an initial upregulation at 30 DPF (25 ng EE2/l: 2.16-fold, $p = 0.0012$) followed by significant suppression at 50 DPF at both concentrations (5 ng EE2/l: − 1.59-fold, $p = 0.0002$; 25 ng EE2/l: − 1.47-fold, $p = 0.0133$). Under 5 ng EE2/l, IGF-I mRNA was still reduced at 75 DPF (− 2.38-fold, $p = 0.0001$) followed by a significant elevation at 100 DPF (1.66-fold, $p = 0.0281$). The ERα mRNA levels tended to be suppressed over the whole treatment phase. At several points in time (5 ng EE2/l at 50 DPF: − 1.53, $p = 0.0152$; 25 ng EE2/l at 100 DPF: − 1.41-fold, $p = 0.0496$), the suppression was significant (Fig. 3d).

Gonad IGF-I and ERα Gene Expression

In male gonads, IGF-I mRNA levels (Fig. 4a) were significantly reduced at 50 and 75 DPF (50 DPF: 5 ng EE2/l: − 3.04-fold, $p = 0.0286$; 25 ng EE2/l: − 6.07-fold, $p = 0.0286$; 75 DPF: 25 ng EE2/l: − 2.33-fold, $p = 0.0303$). Only at 100 DPF, IGF-I mRNA levels returned to control levels (5 ng/l) or were even significantly elevated (25 ng/l). ERα mRNA in testes was suppressed over the whole exposure period. This decrease was statistically significant at 30 DPF under 25 ng EE2/l (− 2.09-fold, $p = 0.0339$) and at 50 (− 2.73-fold, $p = 0.0286$) and 100 DPF (− 1.95-fold, $p = 0.037$) under 5 ng EE2/l (Fig. 4b).

In female gonads, IGF-I mRNA expression was significantly elevated at 100 DPF (5 ng/l: 3.17-fold, $p = 0.0023$; 25 ng EE2/l: 2.55-fold, $p = 0.0042$) (Fig. 4c). ERα mRNA levels did not change throughout the experimental period (Fig. 4d).

Pituitary GH Levels

The amount of GH mRNA was determined using extracts from whole brain with pituitary attached since the pituitaries at

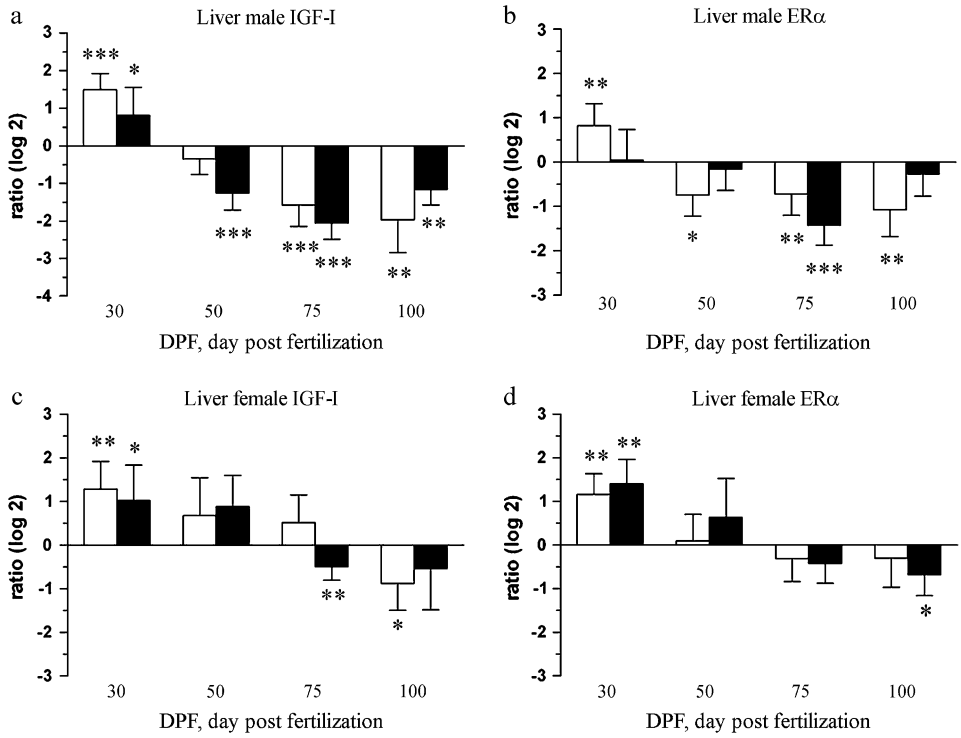

FIG. 2. Influence of EE2 exposure on IGF-I and ERα gene expression in liver (a–d). Relative changes (log2) of IGF-I (a, c) and ERα (b, d) mRNA expression in EE2-exposed tilapia as compared to age-matched control tilapia. White columns: 5 ng EE2/l, black columns: 25 ng EE2/l. The x-axis shows stages in DPF. Bars represent mean values with SD. Significance level: $*p < 0.05$, $**p < 0.005$, and $***p < 0.0005$.

30 and 50 DBF were too small to allow dissection. At 30 DPF, no effect of EE2 exposure on GH expression was observed in both sexes (Fig. 5). In females, after a significant increase in GH mRNA at 50 DPF (5 ng EE2/l: 3.91-fold, $p = 0.002$; 25 ng EE2/l: 2.62-fold, $p = 0.05$), GH mRNA decreased at 100 DPF under 5 ng EE2/l to the threefold ($p = 0.03$) and under 25 ng EE2/l to the 10-fold ($p = 0.03$) (Fig. 5a). In males, GH mRNA exhibited only a trend to similarly increase at 50 DPF but was significantly decreased at 100 DPF under 25 ng EE2/l by the sevenfold ($p = 0.001$) (Fig. 5b).

DISCUSSION

Developmental exposure to EE2 clearly shifted the sex ratio: female and male monosex fish populations exhibited a higher percentage of females after exposure to both 5 and 25 ng EE2/l. The growth rate of the developing tilapia was also suppressed by EE2 treatment. These findings are in agreement with our previous observations on high-dose effects of EE2 (Shved *et al.*, 2007), but the present results indicate that concentrations at environmental levels of EE2 still have the capacity to impair tilapia growth and sexual development.

Our hypothesis was that the growth reduction resulting from estrogen exposure is related to a cross talk between the estrogen and the IGF-I system, be it through an estrogen effect on the liver as source of the circulating (endocrine) IGF-I, or be it an estrogen effect on the paracrine/autocrine IGF-I system in the peripheral organs. Our experimental results show that hepatic IGF-I expression can be suppressed by EE2 exposure. This is particularly the case in males but with prolonged exposure also in females. The decline in hepatic IGF-I mRNA found here in developing tilapia is in agreement with previous investigations in which estrogen exposure of adults of different fish species led to a decrease in hepatic IGF-I mRNA (Carnevali *et al.*, 2005; Filby *et al.*, 2006; Riley *et al.*, 2004). The stronger effect on liver IGF-I of males than of females correlates with a stronger EE2 effect on growth indices in males. This correlation suggests that the EE2-induced growth reduction goes back to the EE2-induced reduction of hepatic IGF-I expression, that is, to an endocrine effect and not to a general toxic effect.

This interpretation is further supported by our findings on GH mRNA, which reacts roughly in parallel to the endocrine hepatic IGF-I system. In females, GH mRNA was significantly raised at 50 DPF but not in males. The different response also may explain the lesser growth impairment found in females when compared to males. At 100 DPF, in both sexes, GH mRNA was significantly downregulated. This decrease in GH mRNA may well be caused by a direct action of EE2 on the pituitary GH cells. Because E2 increased the expression of somatostatin-14 in goldfish brain (Canosa *et al.*, 2002) EE2

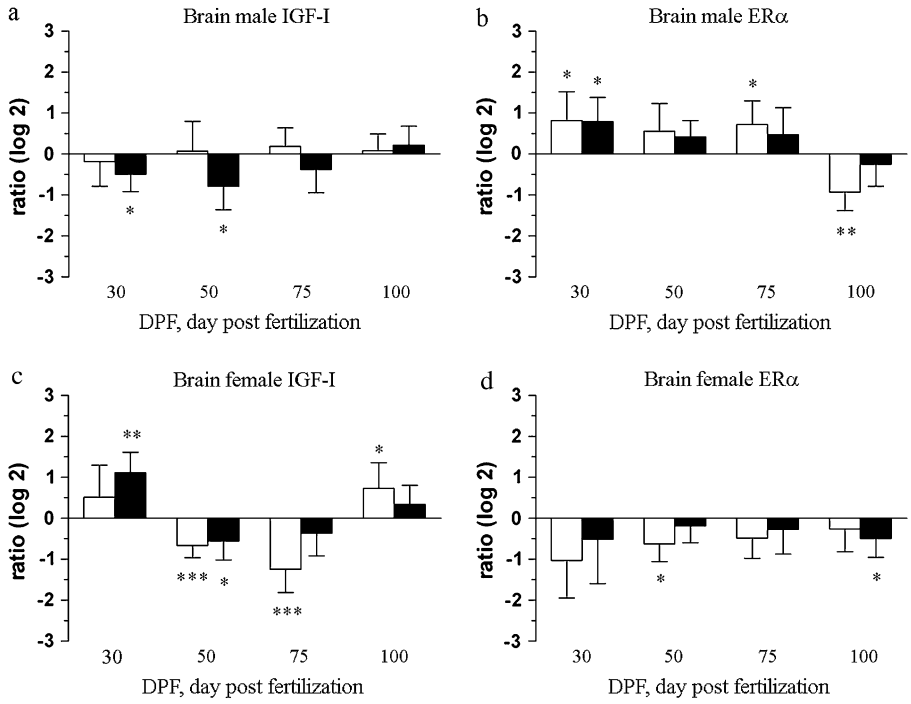

FIG. 3. Influence of EE2 exposure on IGF-I and ERα gene expression in brain (a–d). Relative changes (log2) of IGF-I (a, c) and ERα (b, d) mRNA expression in EE2-treated as compared to age-matched control tilapia. White columns: 5 ng EE2/l, black columns: 25 ng EE2/l. The x-axis shows stages in DPF. Bars represent mean values with SD. Significance level: $*p < 0.05$, $**p < 0.005$, and $***p < 0.0005$.

may have as well suppressed GH at the hypothalamic level by acting on somatostatin-14.

Very few studies have dealt with effects of estrogens on fish pituitary GH. No effects on GH expression were observed in adult fathead minnow exposed to 35 ng E2/l in surrounding water for 14 days (Filby *et al.*, 2006). However, in steroid-primed immature rainbow trout after E2 injections (Holloway and Leatherland, 1997) and in female goldfish after implantation of pellets containing 100 μg E2/g body weight for 5 days (Zou *et al.*, 1997), a stimulation of GH synthesis and secretion by E2 has been reported. Similarly, *in vitro* treatment of rainbow trout pituitary glands with 1000nM of the xenoestrogen *o,p′*-dichlorodiphenyltrichloroethane resulted in a significant induction of GH mRNA (Elango *et al.*, 2006). The results of the latter studies reporting an increase in GH mRNA after short-term treatment with (xeno)estrogens are consistent with ours that show the significant increase in GH mRNA in females at 50 DPF and a similar trend in males. While Shved *et al.* (2007) did not investigate early stages, they reported that feeding of tilapia with 125 μg EE2/g food from 10 to 40 DPF led to a significant reduction of pituitary GH mRNA female (75 and 90 DPF) and male (165 DPF) fish. This reduction is compatible with the present results where a significant decrease in GH mRNA occurred after 90 days of EE2 exposure. Some remaining discrepancies between above studies and the present report are probably due to the different estrogenic compounds used, to the different modes of application, or to hormone application at different life stages of the fish.

The question arises as to whether the altered hepatic IGF-I expression in EE2-exposed fish is a direct consequence of the EE2-mediated activation of the ER pathway. To this end, we analyzed the expression of ERα. In our study, estrogen treatment led to a downregulation of hepatic ERα expression what is in contrast to the studies of Filby *et al.* (2006) and Shved *et al.* (2007). In both these studies, estrogen-induced decrease in IGF-I mRNA was accompanied by an upregulation of ERα mRNA. However, a major difference between our study and these two is that we used a long-term exposure regime, while those treated the fish only for 14 and 30 days, respectively. At 30 DPF, that is, after 20 days of exposure, we also observed an upregulation of hepatic ERα, and only thereafter, it turned into a downregulation. Thus, the difference between our results and those of Filby *et al.* (2006) and Shved *et al.* (2007) seems to be due to the exposure duration. The hepatic IGF-I levels showed time-dependent changes that are fairly similar to those of the ERα, with an initial upregulation followed by a downregulation on the longer run. This correlation suggests a mechanistic relationship between the ER pathway and the IGF-I gene expression, but future studies will have to substantiate this hypothesis.

In contrast to the liver, the peripheral organs show quite different responses of ERα and IGF-I mRNA to EE2

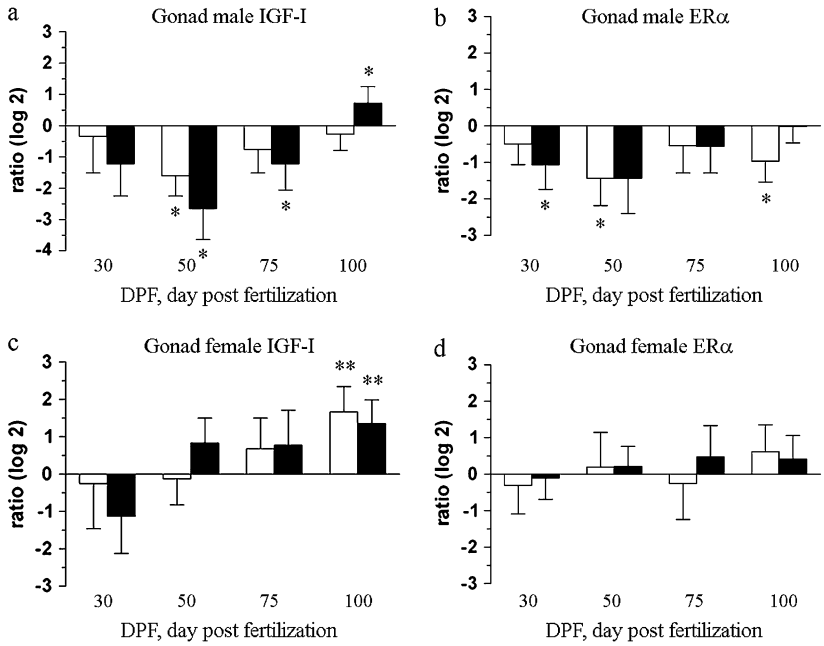

FIG. 4. Influence of EE2 exposure on IGF-I gene expression in male and female gonads (a–d). Relative changes log2 of IGF-I (a, c) and ERα (b, d) mRNA expression in EE2-exposed as compared to age-matched control tilapia. White columns: 5 ng EE2/l, black columns: 25 ng EE2/l. The x-axis shows stages in DPF. Bars represent mean values with SD. Significance level: $*p < 0.05$ and $**p < 0.005$.

treatment suggesting that the mechanisms of the IGF-I-estrogen cross talk differ between the endocrine part of the IGF-I system (liver) and the autocrine/paracrine part (gonads and brain). ERα mRNA in male brain was significantly elevated at 30 DPF at both concentrations to be significantly decreased at the end of the experimental period (100 DPF) under 5 ng EE2/l. In females, brain ERα mRNA tended to be decreased with some significancies all over the experimental period. In contrast, in adult fathead minnow, exposure to 35 ng E2/l for 14 days did not influence ERα mRNA in brain of either sex (Filby *et al*., 2006). Thus, the brain ERα mRNA seems to be downregulated only during long-term developmental exposure. In the same study (Filby *et al*., 2006), IGF-I mRNA in brain was significantly induced in both sexes, the increase being more pronounced in female brain. This is quite consistent with the present data where also a significant upregulation of IGF-I mRNA in female brain was observed after 20 days of EE2 exposure. Later, however, brain IGF-I mRNA was downregulated, the effect being stronger in females.

Developmental exposure to low concentrations of EE2 has also consequences on the IGF-I expression in organs where this hormone is considered to have an autocrine or paracrine function such as brain and gonads. The response patterns in these organs were different to the liver. However, also here a sex-specific response pattern was indicated, at least in the brain as outlined above. In the testis, IGF-I showed a significant but transitory downregulation which disappeared or even turned into an induction under prolonged exposure. In contrast, in the ovaries of exposed fish, IGF-I mRNA levels showed an increase becoming significant at 100 DPF. The functions of IGF-I include roles in spermatogenesis and oocyte proliferation and maturation. In Japanese eel and tilapia cultured testes, IGF-I stimulated spermatogenesis induced by 11-ketosterone (Nader *et al*., 1999; Tokalov and Gutzeit, 2005). In rainbow trout testes, IGF-I mRNA increased after GH treatment (Biga *et al*., 2004; Le Gac *et al*., 1996) and GH and IGF-I stimulated the incorporation of thymidine into spermatogonia and primary spermatocytes (Loir, 1999). In the ovary of different fish species, IGF-I stimulated thymidine incorporation in vitellogenic follicles (Srivastava and Van der Kraak, 1994), promoted oocyte maturation (e.g., Lokman *et al*., 2007; Negatu *et al*., 1998), and selectively influenced the production of sex steroids (e.g., Chourasia and Joy, 2007; Weber *et al*., 2007). In sturgeon, previtellogenic follicles exhibited higher IGF-I and IGF-I receptor (IGF-1R) mRNA levels in maturing females than in age-matched non-maturing females, and females entering vitellogenesis showed an increase in ovarian IGF-I and IGF-1R mRNA (Wuertz *et al*., 2007), thus emphasizing the importance of local IGF-I. Recent evidence obtained in zebra fish exposed to 10 ng EE2/l surrounding water indicated not only a reduction of juvenile growth but also an impairment of sexual maturity, adult fecundity, and fertilization success (Schäfers *et al*., 2007). Similarly, the exposure of Japanese medaka to environmentally relevant concentration of 1.6–157 ng/l E2 impaired fecundity and reduced the number of spawns (Jukosky *et al*., 2008). Whether the EE2 effect on IGF-I expression in gonads of developing tilapia is involved in the

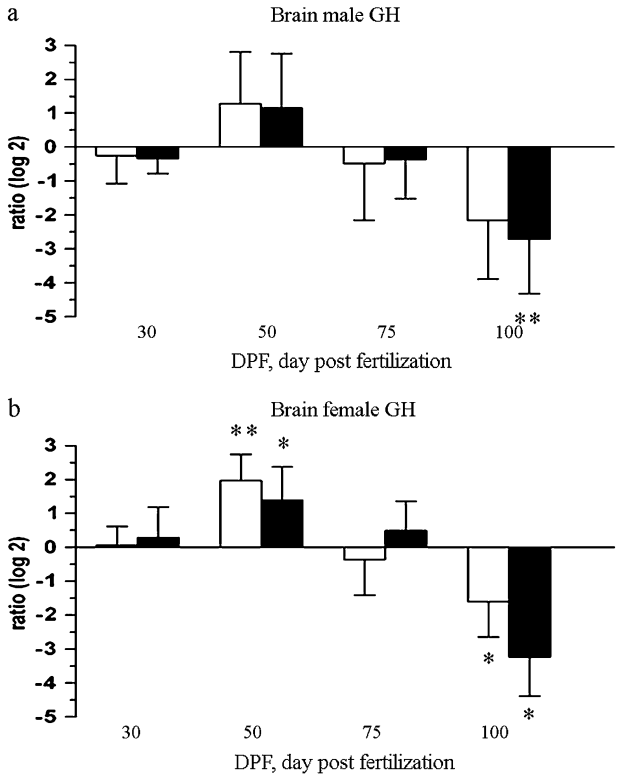

FIG. 5. Influence of EE2 exposure on IGF-I gene expression in male (a) and female (b) brain. Relative changes log2 of IGF-I mRNA expression in EE2-exposed as compared to age-matched control tilapia. White columns: 5 ng EE2/l, black columns: 25 ng EE2/l. The x-axis shows stages in DPF. Bars represent mean values with SD. Significance level: $*p < 0.05$ and $**p < 0.005$.

feminization effect of the experimental treatment cannot be asserted from the present data; however, it is clear that EE2 changes normal expression patterns of IGF-I in gonad ontogeny and thereby has the potential to lead to adverse consequences.

Our study advances endocrine disruptor research in that it extends the targets for action of EDCs beyond the reproductive axis—which has received most attention to date. Importantly, we could demonstrate that such effects occur at low concentrations similar to environmental levels. Individual growth has consequences for demographic parameters such as age-specific survival, time to maturation, or fecundity. Thus, our findings obtained with low doses of EE2 point to the impact of the GH/IGF-I system, through which environmental estrogens, in addition to their direct effect on fish reproduction, could alter population growth of fish species. Future research has to address the mechanisms through which estrogens act to modulate the GH/IGF-I system. In particular, given our findings on the correlation between ER and IGF-I changes, it would be important to examine whether the EE2 effects on the GH/IGF-I system in liver and extrahepatic sites are mediated through the ER pathway and/or through other mechanisms.

FUNDING

The Swiss National Research Foundation (NRP 50, project 4050-66580).

REFERENCES

Agresti, A., and Coull, B. A. (1998). Approximate is better than "exact" for interval estimation of binomial proportions. *Am. Statist.* **52,** 119–126.

Arsenault, J. T., Fairchild, W. L., MacLatchy, D. L., Burridge, L., Haya, K., and Brown, S. B. (2004). Effects of water-borne 4-nonylphenol and 17beta-estradiol exposures during parr-smolt transformation on growth and plasma IGF-I of Atlantic salmon (*Salmo salar L.*). *Aquat. Toxicol.* **66,** 255–265.

Ashfield, L. A., Pottinger, T. G., and Sumpter, J. P. (1998). Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. *Environ. Toxicol. Chem.* **17,** 679–686.

Baroiller, J. F., Chourrout, D., Fostier, A., and Jalabert, B. (1995). Temperature and sex chromosomes govern sex ratios of the mouthbrooding cichlid fish *Oreochromis niloticus*. *J. Exp. Zool.* **273,** 216–223.

Baroiller, J. F., Guiguen, Y., and Fostier, A. (1999). Endocrine and environmental sex differentiation in fish. *Cell. Mol. Life Sci.* **55,** 910–931.

Belfroid, A. C., Van der Horst, A., Vethaak, A. D., Schafer, A. J., Rijs, G. B. J., Wegener, J., and Cofino, W. P. (1999). Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Sci. Total Environ.* **225,** 101–108.

Ber, R., and Daniel, V. (1992). Structure and sequence of the growth hormone-encoding gene from *Tilapia nilotica*. *Gene* **113,** 245–250.

Berishvili, G., D'Cotta, H., Baroiller, J.-F., Segner, H., and Reinecke, M. (2006a). Differential expression of IGF-I mRNA and peptide in the male and female gonad during early development of a bony fish, the tilapia *Oreochromis niloticus*. *Gen. Comp. Endocrinol.* **146,** 204–210.

Berishvili, G., Shved, N., Eppler, E., Clota, F., Baroiller, J.-F., and Reinecke, M. (2006b). Organ-specific expression of IGF-I during early development of bony fish as revealed in the tilapia, *Oreochromis niloticus*, by in situ hybridisation and immunohistochemistry: Indication for the particular importance of local IGF-I. *Cell Tissue Res.* **325,** 287–301.

Biga, P. R., Schelling, G. T., Hardy, R. W., Cain, K. D., Overturf, K., and Ott, T. L. (2004). The effects of recombinant bovine somatotropin (rbST) on tissue IGF-I, IGF-I receptor, and GH mRNA levels in rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.* **135,** 324–333.

Caelers, A., Berishvili, G., Meli, M. L., Eppler, E., and Reinecke, M. (2004). Establishment of a real-time RT-PCR for the determination of absolute amounts of IGF-I and IGF-II gene expression in liver and extrahepatic sites of the tilapia. *Gen. Comp. Endocrinol.* **137,** 196–204.

Canosa, L. F., Lin, X., and Peter, R. E. (2002). Regulation of expression of somatostatin genes by sex steroid hormones in goldfish forebrain. *Neuroendocrinology* **76,** 8–17.

Cargouët, M., Perdiz, D., Mouatassim-Souali, A., Tamisier-Karolak, S., and Levi, Y. (2004). Assessment of river contamination by estrogenic compounds in Paris area (France). *Sci. Total Environ.* **324,** 55–66.

Carnevali, O., Cardinali, M., Maradonna, F., Parisi, M., Olivotto, I., Polzonetti-Magni, A. M., Mosconi, G., and Funkenstein, B. (2005). Hormonal regulation of hepatic IGF-I and IGF-II gene expression in the marine teleost *Sparus aurata*. *Mol. Reprod. Dev.* **71,** 12–18.

Chang, X. T., Kobayashi, T., Todo, T., Ikeuchi, T., Yoshiura, Y., Kajiura-Kobayashi, H., Morrey, C., and Nagahama, Y. (1999). Molecular cloning of estrogen receptors alpha and beta in the ovary of a teleost fish, the tilapia (*Oreochromis niloticus*). *Zool. Sci.* **16,** 653–658.

Chen, C. Y., Wen, T. Y., Wang, G. S., Cheng, H. W., Lin, Y. H., and Lien, G. W. (2007). Determining estrogenic steroids in Taipei waters and removal in drinking water treatment using high-flow solid-phase extraction and liquid chromatography/tandem mass spectrometry. *Sci. Total Environ.* **378,** 352–365.

Chourasia, T. K., and Joy, K. P. (2008). Estrogen-2/4-hydroxylase activity is stimulated during germinal vesicle breakdown induced by hCG, IGF-1, GH and insulin in the catfish *Heteropneustes fossilis*. *Gen. Comp. Endocrinol.* **155,** 413–421.

Davis, L. K., Hiramatsu, N., Hiramatsu, K., Reading, B. J., Matsubara, T., Hara, A., Sullivan, C. V., Pierce, A. L., Hirano, T., and Grau, E. G. (2007). Induction of three vitellogenins by 17beta-estradiol with concurrent inhibition of the growth hormone-insulin-like growth factor 1 axis in a euryhaline teleost, the tilapia (*Oreochromis mossambicus*). *Biol. Reprod.* **77,** 614–625.

D'Cotta, H., Fostier, A., Guiguen, Y., Govoroun, M., and Baroiller, J. F. (2001). Search for genes involved in the temperature-induced gonadal sex differentiation in the tilapia, *Oreochromis niloticus*. *J. Exp. Zool.* **290,** 574–585.

Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P., and Waldock, M. (1998). Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ. Sci. Technol.* **32,** 1549–1558.

Dethlefsen, V., von Westernhagen, H., and Cameron, P. (1996). Malformations in the North Sea pelagic fish embryos during the period 1984–1995. *ICES J. Mar. Sci.* **53,** 1024–1035.

Devlin, R. H., and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* **208,** 191–364.

Elango, A., Shepherd, B., and Chen, T. T. (2006). Effects of endocrine disrupters on the expression of growth hormone and prolactin mRNA in the rainbow trout pituitary. *Gen. Comp. Endocrinol.* **145,** 116–127.

Eppler, E., Caelers, A., Shved, N., Hwang, G., Rahman, A. M., Maclean, N., Zapf, J., and Reinecke, M. (2007). Insulin-like growth factor I (IGF-I) in a growth-enhanced transgenic (GH-overexpressing) bony fish, the tilapia (*Oreochromis niloticus*): Indication for a higher impact of autocrine/paracrine than of endocrine IGF-I. *Transgenic Res.* **16,** 479–489.

Filby, A. L., Thorpe, K. L., Maack, G., and Tyler, C. R. (2007). Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. *Aquat. Toxicol.* **81,** 219–231.

Filby, A. L., Thorpe, K. L., and Tyler, C. R. (2006). Multiple molecular effect pathways of an environmental oestrogen in fish. *J. Mol. Endocrinol.* **37,** 121–134.

Game, C., Gagnon, M. M., Webb, D., and Lim, R. (2006). Endocrine disruption in male mosquitofish (*Gambusia holbrooki*) inhabiting wetlands in Western Australia. *Ecotoxicology.* **15,** 665–672.

Gross-Sorokin, M. Y., Roast, S. D., and Brighty, G. C. (2006). Assessment of feminization of male fish in English rivers by the Environment Agency of England and Wales. *Environ. Health Perspect.* **114** (Suppl. 1), 147–151.

Holloway, A. C., and Leatherland, J. F. (1997). Effect of gonadal steroid hormones on plasma growth hormone concentrations in sexually immature rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.* **105,** 246–254.

Hwang, G. L., Rahman, A. M., Razak, A. S., Sohm, F., Farahmand, H., Smith, A., Brooks, C., and Maclean, N. (2003). Isolation and characterisation of tilapia beta-actin promoter and comparison of its activity with carp beta-actin promoter. *Biochim. Biophys. Acta.* **1625,** 11–18.

Jones, J. I., and Clemmons, D. R. (1995). Insulin-like growth factors and their binding proteins: Biological actions. *Endocr. Rev.* **16,** 3–34.

Jukosky, J. A., Watzin, M. C., and Leiter, J. C. (2008). The effects of environmentally relevant mixtures of estrogens on Japanese medaka (*Oryzias latipes*) reproduction. *Aquat. Toxicol.* **86,** 323–331.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., and Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: A national reconnaissance. *Environ. Sci. Technol.* **36,** 1202–1211.

Kuch, H. M., and Ballschmiter, K. (2001). Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. *Environ. Sci. Technol.* **35,** 3201–3206.

Lavado, R., Thibaut, R., Raldúa, D., Martín, R., and Porte, C. (2004). First evidence of endocrine disruption in feral carp from the Ebro River. *Toxicol. Appl. Pharmacol.* **19,** 247–257.

Le Gac, F., Loir, M., Le Bail, P..-Y., and Ollitrault, M. (1996). Insulin-like growth factor I (IGF-I) mRNA and IGF-I receptor in trout testis and in isolated spermatogenic and Sertoli cells. *Mol. Reprod. Dev.* **44,** 23–35.

Lerner, D. T., Björnsson, B. T., and McCormick, S. D. (2007). Larval exposure to 4-nonylphenol and 17beta-estradiol affects physiological and behavioral development of seawater adaptation in Atlantic salmon smolts. *Environ. Sci. Technol.* **41,** 4479–4485.

Loir, M. (1999). Spermatogonia of rainbow trout: II. In vitro study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. *Mol. Reproduct. Dev.* **53,** 434–442.

Lokman, P. M., George, K. A., Divers, S. L., Algie, M., and Young, G. (2007). 11-Ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis*, in vitro. *Reproduction* **133,** 955–967.

Marlatt, V. L., Martyniuk, C. J., Zhang, D., Xiong, H., Watt, J., Xia, X., Moon, T., and Trudeau, V. L. (2008). Auto-regulation of estrogen receptor subtypes and gene expression profiling of 17beta-estradiol action in the neuroendocrine axis of male goldfish. *Mol. Cell. Endocrinol.* **283,** 38–48.

McCormick, S. D., O'Dea, M. F., Moeckel, A. M., Lerner, D. T., and Bjornsson, B. T. (2005). Endocrine disruption of parr-smolt transformation and seawater tolerance of Atlantic salmon by 4-nonylphenol and 17ß-estradiol. *Gen. Comp. Endocrinol.* **142,** 280–288.

Menuet, A., Le Page, Y., Torres, O., Kern, L., Kah, O., and Pakdel, F. (2004). Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERalpha, ERbeta1 and ERbeta2. *J. Mol. Endocrinol.* **32,** 975–986.

Milston, R. H., Fitzpatrick, M. S., Vella, A. T., Clements, S., Gundersen, D., Feist, G., Crippen, T. L., Leong, J., and Schreck, C. B. (2003). Short-term exposure of Chinook salmon (*Oncorhynchus tshawytscha*) to o,p-DDE or DMSO during early life history stages causes long-term humoral immunosuppression. *Environ. Health Perspect.* **111,** 1601–1607.

Nader, M. R., Miura, T., Ando, N., Miura, C., and Yamauchi, K. (1999). Recombinant human insulin-like growth factor I stimulates all stages of 11-ketotestosterone-induced spermatogenesis in the Japanese eel, *Anguilla japonica*, in vitro. *Biol. Reprod.* **61,** 944–947.

Negatu, Z., Hsiao, S. M., and Wallace, R. A. (1998). Effects of insulin-like growth factor-I on final oocyte maturation and steroid production in *Fundulus heteroclitus*. *Fish Physiol. Biochem.* **19,** 13–21.

Perrot, V., Moiseeva, E. B., Gozes, Y., Chan, S. J., Ingleton, P., and Funkenstein, B. (1999). Ontogeny of the insulin-like growth factor system (IGF-I, IGF-II, and IGF-IR) in gilthead seabream (*Sparus aurata*): Expression and cellular localization. *Gen. Comp. Endocrinol.* **116,** 445–460.

Radaelli, G., Domeneghini, C., Arrighi, S., Bosi, G., Patruno, M., and Funkenstein, B. (2003). Localization of IGF-I, IGF-I receptor, and IGFBP-2 in developing *Umbrina cirrosa* (Pisces: Osteichthyes). *Gen. Comp. Endocrinol.* **130,** 232–244.

Reinecke, M., and Collet, C. (1998). The phylogeny of the insulin-like growth factors. *Int. Rev. Cytol.* **183,** 1–94.

Reinecke, M., Bjornsson, B. T., Dickhoff, W. W., McCormick, S. D., Navarro, I., Power, D. M., and Gutierrez, J. (2005). Growth hormone and insulin-like growth factors in fish: Where we are and where to go. *Gen. Comp. Endocrinol.* **142,** 20–24.

Reinecke, M., Schmid, A., Ermatinger, R., and Loffing-Cueni, D. (1997). Insulin-like growth factor I in the teleost *Oreochromis mossambicus*, the tilapia: Gene sequence, tissue expression, and cellular localization. *Endocrinology* **138,** 3613–3619.

Riley, L. G., Hirano, T., and Grau, E. G. (2004). Estradiol-17beta and dihydrotestosterone differentially regulate vitellogenin and insulin-like growth factor-I production in primary hepatocytes of the tilapia *Oreochromis mossambicus*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **138,** 177–186.

Schäfers, C., Teigeler, M., Wenzel, A., Maack, G., Fenske, M., and Segner, H. (2007). Concentration- and time-dependent effects of the synthetic estrogen, 17alpha-ethinylestradiol, on reproductive capabilities of the zebrafish, *Danio rerio*. *J. Toxicol. Environ. Health A.* **70,** 768–779.

Shved, N., Berishvili, G., D'Cotta, H., Baroilller, J. F., Segner, H., Eppler, E., and Reinecke, M. (2007). Ethinylestradiol (EE2) differentially interferes with insulin-like growth factor-I (IGF-I) in liver and extrahepatic sites during development of male and female bony fish. *J. Endocrinol.* **195,** 513–523.

Srivastava, R. K., and Van der Kraak, G. (1994). Regulation of DNA-synthesis in goldfish ovarian follicles by hormones and growth factors. *J. Exp. Zool.* **270,** 263–272.

Sumpter, J. P., and Jobling, S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.* **103**(Suppl. 7), 173–178.

Ternes, T. A., Kreckel, P., and Mueller, J. (1999). Behaviour and occurrence of estrogens in municipal sewage treatment plants—II. Aerobic batch experiments with activated sludge. *Sci. Total Environ.* **225,** 91–99.

Tokalov, S. V., and Gutzeit, H. O. (2005). Spermatogenesis in testis primary cell cultures of the tilapia (*Oreochromis niloticus*). *Dev. Dyn.* **233,** 1238–1247.

Vong, Q. P., Chan, K. M., and Cheng, C. H. (2003). Quantification of common carp (*Cyprinus carpio*) IGF-I and IGF-II mRNA by real-time PCR: Differential regulation of expression by GH. *J. Endocrinol.* **178,** 513–521.

Weber, G. M., Moore, A. B., and Sullivan, C. V. (2007). In vitro actions of insulin-like growth factor-I on ovarian follicle maturation in white perch (*Morone americana*). *Gen. Comp. Endocrinol.* **151,** 180–187.

Wood, A. W., Duan, C., and Bern, H. A. (2005). Insulin-like growth factor signaling in fish. *Int. Rev. Cytol.* **243,** 215–285.

Wuertz, S., Gessner, J., Kirschbaum, F., and Kloas, W. (2007). Expression of IGF-I and IGF-I receptor in male and female sterlet, *Acipenser ruthenus*—evidence for an important role in gonad maturation. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **147,** 223–230.

Zou, J. J., Trudeau, V. L., Cui, Z., Brechin, J., Mackenzie, K., Zhu, Z., Houlihan, D. F., and Peter, R. (1997). Estradiol stimulates growth hormone production in female goldfish. *Gen. Comp. Endocrinol.* **106,** 102–112.